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*Proceeding Paper*

# Effect of Cryoprotectants on the Survival Rate of Freeze-Dried *Limosilactobacillus frumenti* and Their Application in Cucumber Fermentation †

**Marinich Net [1,2], Sophak Phourng [2], Dolla Bros [2], Socheata Mao [1,3], Yve Wache [3] and Reasmey Tan [1,2,*]**

[1] Research and Innovation Center, Institute of Technology of Cambodia, Russian Federation Blvd., Phnom Penh P.O. Box 86, Cambodia; net.marinich@itc.edu.kh (M.N.); socheata.mao@agrosupdijon.fr (S.M.)
[2] Faculty of Chemical and Food Engineering, Institute of Technology of Cambodia, Russian Federation Blvd., Phnom Penh P.O. Box 86, Cambodia; sophakphourng@gmail.com (S.P.); brosdolla1306@gmail.com (D.B.)
[3] Université Bourgogne Europe, Institut Agro, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), L'Unité Mixte de Recherche Procédés Alimentaires et Microbiologiques (UMR PAM), F-21000 Dijon, France; yves.wache@agrosupdijon.fr
* Correspondence: rtan@itc.edu.kh; Tel.: +855-12-602-202
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**Abstract**

Cryoprotectants are used to protect biological cells from damage caused by freezing. This study aimed to determine the effect of various types of cryoprotectant on the survival rate of freeze-dried *Limosilactobacillus frumenti* (*L. frumenti*) used as a starter culture for cucumber fermentation. Mixtures of freeze-dried *L. frumenti* with cryoprotectants were prepared using two different ratios (1:2 and 1:10). The survival rate of *L. frumenti* was determined by viable cell counts (CFU/mL) after freeze-drying, and fermentation performance was evaluated in terms of physicochemical quality and sensory evaluation. Skim milk proved to be the most effective cryoprotectant, yielding a survival rate of approximately 70% (70.07% for the 1:10 ratio and 70.01% for the 1:2 ratio) after 24 h of storage at 4 °C. Sensory evaluation indicated that cucumber fermentation prepared with freeze-dried *L. frumenti* mixed with skim milk (ratio 1:10) was the most preferred by panelists.

**Keywords:** cryoprotectants; freeze-drying; lactic acid bacteria; cucumbers

## 1. Introduction

Fermentation is a traditional preservation technique widely used to enhance sensory attributes and nutritional quality, especially to prolong the shelf-life of food products. The process relies on the metabolic activity of bacteria, yeasts, or molds to degrade food components into organic acids and inhibit the growth of spoilage microorganisms [1]. Among these, lactic acid bacteria (LAB) play an important role in vegetable fermentation due to the ability to improve flavor and functional properties. The application of LAB as starter cultures in the food industry depends on preservation methods to ensure long-term stability in terms of viability and functional activity [2]. However, during the dehydration process, LAB cells are exposed to multiple environmental stresses such as freezing, drying, prolonged exposure to low water activity, and rehydration. These stresses can significantly reduce cell survival. In general, LAB survival throughout the dehydration process is affected by several factors, including strain-specific stress tolerance, initial cell concentration, growth and drying conditions, composition of protective agents, freezing

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rate, storage conditions (temperature, atmosphere, and relative humidity), and rehydration conditions [3]. To preserve the viable LAB, there are three main techniques that can be used comprising subculturing, low-temperature preservation (cryopreservation), and freeze-drying [4]. Among these methods, freeze-drying is the most commonly used for preserving LAB cultures. Freeze-drying, also known as lyophilization, is used to maintain the nutritional quality, sensory attributes and structural qualities of fermented vegetables by removing water through sublimation at low temperatures, thereby minimizing chemical and microbial activity. However, processing conditions greatly affect product quality and cell survival [5]. Therefore, the use of effective cryoprotectants is important for maintaining cell viability and ensuring an effective fermentation process by preventing mechanical damage caused by ice crystal formation and reducing osmotic stress through the stabilization of residual water [6]. The objective of this study is to evaluate and identify the most effective cryoprotectant for LAB survival and cucumber fermentation quality.

## 2. Materials and Methods

### *2.1. Experimental Design*

Seven types of cryoprotectant, including saline (CPT1), skim milk (CPT2), sucrose (CPT3), skim milk mixed with sucrose (CPT4), maltodextrin (CPT5), lactose (CPT6), and glucose (CPT7), were purchased from Watermech Technologies Co., Ltd., Phnom Penh, Cambodia. Freeze-dried *L. frumenti* was prepared with different cryoprotectants using different ratios such as 1:2 and 1:10. Following the identification of the most effective cryoprotectant, the selected cryoprotectant was applied in the cucumber fermentation process to compare the result with pure culture and spontaneous fermentation.

### *2.2. Cryoprotectant Preparation*

A measurement of 10 g of each cryoprotectant was mixed with 100 mL of distilled water and was autoclaved at 121 °C for 15 min except the skim milk. The final concentration of cell suspensions was adjusted to 1% (*m*/*v*) and vortexed for homogeneity.

### *2.3. LAB Freeze-Dried Powder Preparation*

The *L. frumenti* was previously isolated from commercial fermented cucumber and maintained at the Food Microbiology Laboratory, Institute of Technology of Cambodia. The stain was stored at −80 °C in a 40% (*v*/*v*) glycerol stock. The collected strain was streaked onto de Man, Rogosa and Sharpe (MRS) agar and was incubated under anaerobic conditions at 30 °C for 48 h. After incubation, four to five well-isolated colonies were selected and inoculated into MRS broth, followed by overnight incubation at 30 °C. The cultured cells were collected by centrifugation at 4000 rpm for 10 min at 4 °C and washed twice with sterile 0.9% (*w*/*v*) saline solution. The washed cell pellets were resuspended and mixed with different cryoprotectants at various ratios prior to freeze-drying. Then, 0.5 mL of the LAB suspension was collected prior to freeze-drying, and the remaining suspensions were frozen before lyophilization. The resulting *L. frumenti* was divided for use as a starter culture in cucumber fermentation [7].

### *2.4. Determination of Freeze-Drying LAB Survival Rate*

The survival rate of *L. frumenti* was investigated during storage for 24 h at 4 °C in terms of cell viability. First, 1 g of sample was rehydrated in 2 mL of MRS broth and left for 10min. The rehydrated suspension was then diluted using sterilized saline solution (0.9% *w*/*v*), and $10^{-4}$ and $10^{-5}$ dilutions were spread onto MRS agar. The plates were then incubated at 30 °C for 48 h and the number of cell viable bacteria (CUF/mL) was

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determined using the plate colony counting method to calculate the survival rate as in the below formula [8].

$$\text{Survival rate} = \frac{N_f}{N_i} \times 100 \quad (1)$$

where $N_i$ is the number of colonies count before freeze-drying and $N_f$ is the number of colonies count after freeze-drying.

### 2.5. Cucumber Fermentation Process

The same size young cucumbers were first washed with tap water and blanched at 80 °C for 5 min. The water was then drained out and cucumbers were placed into sterile jars. Afterward, 4% (*w*/*v*) brine solution was prepared and cooled prior to use. The brine was then inoculated under four different conditions, such as spontaneous fermentation, $14.3 \times 10^7$ CFU/mL of *L. frumenti* treated with skim milk (ratio 1:2), $16.3 \times 10^8$ CFU/mL of *L. frumenti* treated with skim milk (ratio 1:10), and pure culture. Each mixture was then added in the same jars containing cucumbers. Fermentation was finally carried out at room temperature for 2 days under anaerobic conditions.

### 2.6. Determination of Physicochemical Parameters

Moisture content was determined using a moisture analyzer (MOC63u, Unibloc, Bolney, West Sussex, UK), and water activity was measured with a water activity meter (AquaLab 4TE, benchtop, Meter Group, Inc., Pullman, WA, USA)). The pH was tested using a pH meter (HI2020, Hanna, Woonsocket, RI, USA), while total titratable acidity was investigated by the titration method. Reducing sugars were analyzed using the dinitrosalicylic acid (DNS) method [9]. Total soluble solids were measured using a digital refractometer (PAL-1, ATAGO, Tokyo, Japan), and salt content was determined using a salt meter (ES-421, ATAGO, Tokyo, Japan).

### 2.7. Sensory Evaluation

Sensory evaluation was conducted to determine consumer preference in terms of flavor, texture, odor, aroma, color, saltiness, sourness, and overall acceptability. A total of 50 panelists assessed the sensory quality of the sample, and the panelists were asked to provide scores in a range of 1 to 9 (1 = Extremely Dislike and 9 = Extremely Like).

### 2.8. Statistical Analysis

The survival rate, moisture content, and water activity of freeze-dried LAB treated with different cryoprotectants were analyzed using one-way ANOVA (IBM® SPSS Statistics version 29 (SPSS Inc., Chicago, IL, USA) followed by Tukey's post hoc test to determine significant differences among mean values ($p < 0.05$). The physicochemical properties of fermented cucumbers were evaluated using the same statistical approach. All experiments were carried out in completely independent triplicates (n = 3), except for moisture content and water activity measurements.

## 3. Results and Discussion

### 3.1. Survival Rate of Limosilactobacillus frumenti

The effects of different cryoprotectant types and cell-to-cryoprotectant ratios on the survival rate of freeze-dried *L. frumenti* are shown in Figure 1. The survival rate of freeze-dried *L. frumenti* differed significantly among cryoprotectant types ($p < 0.05$). Based on the result, the freeze-dried *L. frumenti* in skimmed milk (CPT2) exhibited the highest survival rate of around 70%, followed by sucrose (CPT3), skim milk mixed with sucrose (CPT4), maltodextrin (CPT5), lactose (CPT6), and glucose (CPT7), which revealed progressively lower protective effects. Moreover, the freeze-dried *L. frumenti* in saline (CPT1) had the

lowest survival rate of around 19%, indicating poor cryoprotective performance. However, there were no significant differences observed between the ratios within the same cryoprotectant ($p > 0.05$). These findings indicated that the presence of sugars, such as skimmed milk, sucrose, maltodextrin, lactose and glucose, in the freeze-drying media provided the best protection to the cell membranes during the freeze-drying process [10]. This suggests that these sugars act as effective cryoprotectants, while saline is not a good cryoprotectant for maintaining cell viability.

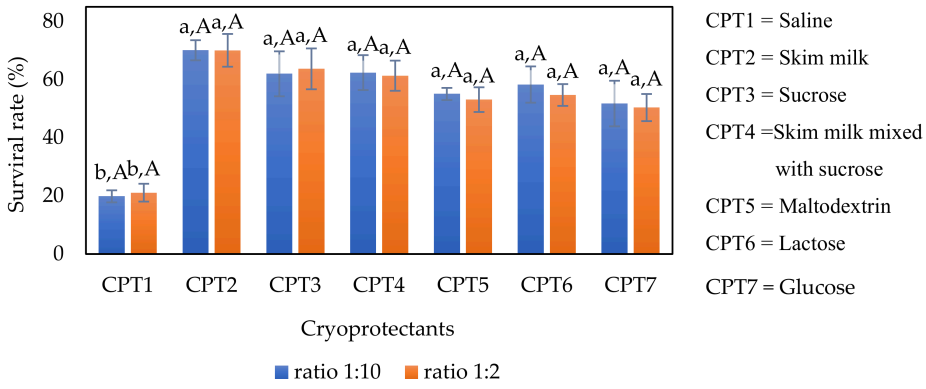



**Figure 1.** Survival rate of freeze-dried *L. frumenti* during storage for 24 h at 4 °C. Graph bars with lower letters (a, b) define the significant difference between each cryoprotectant type, whereas a capital letter (A) defines the significant difference between two ratios ($p < 0.05$).

### *3.2. Moisture Content of Freeze-Dried LAB*

The moisture content of freeze-dried *L. frumenti* powders was significantly influenced by the type of cryoprotectant used ($p < 0.05$), whereas the mixing ratio showed no significant effect within the same cryoprotectant ($p > 0.05$) as shown in Figure 2. Among the tested cryoprotectants, the sucrose (CPT3) sample exhibited the highest moisture contents of 5.53% and 5.6% for ratios 1:10 and 1:2, which are attributed to the hygroscopic nature and strong water binding capacity during the freeze-drying process. However, the saline (CPT1) sample resulted in the lowest moisture content of 2.2% for both ratios, suggesting dehydration efficiency and weaker water matrix interactions, which are desirable for the long-term stability of dried starter cultures [11]. The moisture content of freeze-dried *L. frumenti* should be maintained below 5% for stability and to prevent the loss of cell viability during storage [12]. These results indicated that cryoprotectant types play a more important role than cryoprotectant ratios in the determination of the moisture content of freeze-dried *L. frumenti*. In addition, the insignificant difference between ratios shows that lower concentrations may be sufficient to achieve comparable drying performance.

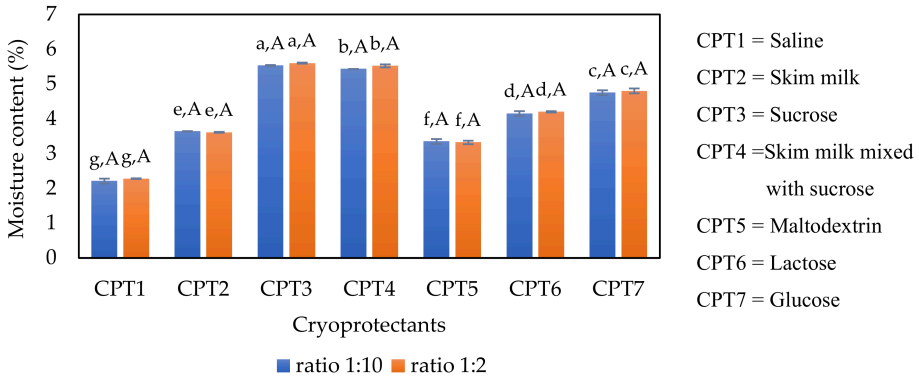



**Figure 2.** Moisture content of freeze-dried *L. frumenti*. Graph bars with lower superscript letters (a–g) define the significant difference between each cryoprotectant type, whereas capital letter (A) defines the significant difference between two ratios ($p < 0.05$).

### *3.3. Water Activity of Freeze-Dried LAB*

Figure 3 demonstrates the water activity of freeze-dried *L. frumenti*. According to the result, both factors, including the different types of cryoprotectant used and the ratios, had no significant influence ($p > 0.05$) on the water activity of freeze-dried *L. frumenti*. Among all treatments, the sucrose (CPT3) sample exhibited the highest water activity value at both ratios, while the freeze-dried *L. frumenti* in skimmed milk (CPT2) showed the lowest. The optimum water activity generally depends on the storage conditions and species of bacteria [13]. In this study, the water activity below 0.3 indicated a threshold associated with reduced biochemical activity and improved storage stability of freeze-dried *L. frumenti* [14,15].

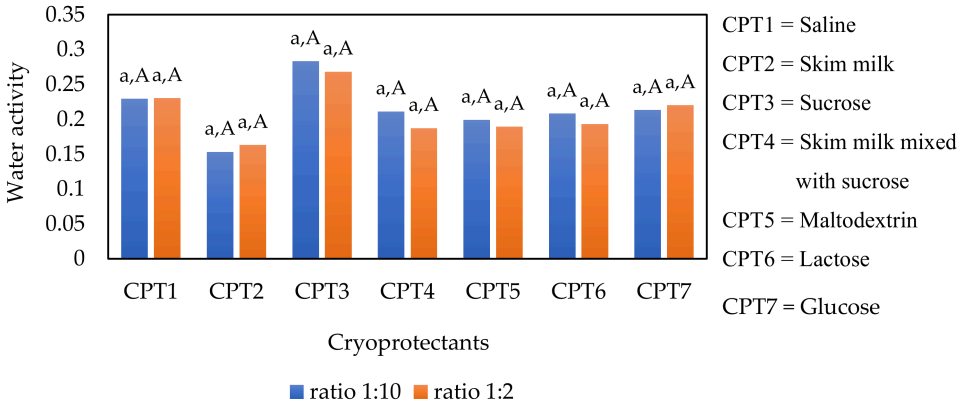



**Figure 3.** Water activity of freeze-dried *L. frumenti*. Graph bars with lower superscript letter (a) define the significant difference between each cryoprotectant type, whereas capital letter (A) defines the significant difference between two ratios ($p < 0.05$).

### *3.4. Physicochemical Characteristics After 48 h Cucumber Fermentation*

The results of physicochemical parameters of fermented cucumbers during 48 h fermentation are summarized in Table 1. The pH values ranged from 3.34 to 3.48, with samples fermented using skim milk (1:2 and 1:10) showing significantly lower pH values compared to the pure culture and spontaneous fermentation ($p < 0.05$). This indicates enhanced acidification efficiency, likely due to the improved cell viability and metabolic activity of LAB preserved with skim milk. Lower pH values are desirable in vegetable fermentation to contribute to microbial safety and product stability because only LAB are able to survive under low acidic conditions [16,17]. Total acidity values did not differ significantly among treatments ($p > 0.05$), suggesting that lactic acid production reached a comparable level across all fermentation processes. This indicates that while acidification kinetics may differ initially, the final acid accumulation converges at later fermentation stages [18]. Reducing the sugar content showed significant differences among treatments ($p < 0.05$). The spontaneous fermentation exhibited the highest reducing sugar level, whereas the pure culture and skim milk-protected LAB treatments showed markedly lower sugar concentrations. This reflects more efficient carbohydrate utilization by inoculated LAB, particularly when cells were protected with skim milk during preservation, leading to enhanced fermentative activity [19]. Total soluble solids and salt content showed no significant differences among treatments ($p > 0.05$). Salt and other solid substances dissolved in the vegetables during fermentation, causing slight changes in the total soluble solid value which indicated that soluble components other than fermentable sugars were minimally affected, while salt content remained consistent due to the controlled formulation. The change in total soluble solids was likely associated with microbial metabolism of sugars into organic acids, alcohols, and other fermentation products. In addition, fermentation increases the activity of enzymes, especially amylases to support sugar breakdown and

decrease soluble sugar concentrations [20]. Overall, the results demonstrated that the use of skim milk as a protective medium for LAB enhances fermentation performance by promoting faster sugar utilization and acidification, without adversely affecting other physicochemical properties. This highlights the potential of skim milk-protected LAB cultures for improving the efficiency and consistency of cucumber fermentation processes.

**Table 1.** Physicochemical parameters of fermented cucumbers during 48 h fermentation.

| LAB | Spontaneous | Skim Milk (1:2) | Skim Milk (1:10) | Pure Culture |
|---|---|---|---|---|
| pH | 3.44 ± 0.02 $^{ab}$ | 3.34 ± 0.014 $^{b}$ | 3.42 ± 0.007 $^{b}$ | 3.48 ± 0.007 $^{a}$ |
| Total acidity (%) | 0.47 ± 0.02 $^{a}$ | 0.47 ± 0.02 $^{a}$ | 0.49 ± 0.02 $^{a}$ | 0.49 ± 0.02 $^{a}$ |
| Reducing sugar (g/L) | 0.59 ± 0.003 $^{a}$ | 0.07 ± 0.0006 $^{bc}$ | 0.12 ± 0.02 $^{b}$ | 0.04 ± 0.003 $^{c}$ |
| Total soluble solids (°Brix) | 4.05 ± 0.07 $^{a}$ | 4.10 ± 0.00 $^{a}$ | 4.05 ± 0.07 $^{a}$ | 4.25 ± 0.07 $^{a}$ |
| Salt content (%) | 2.16 ± 0.007 $^{a}$ | 2.32 ± 0.007 $^{a}$ | 2.23 ± 0.02 $^{a}$ | 2.36 ± 0.02 $^{a}$ |

Each value is presented as mean ± standard deviation. a–c mean with the different letters within the same row are significantly different ($p < 0.05$).

### *3.5. Result of Sensory Evaluation*

The sensory evaluation of fermented cucumbers is demonstrated in Figure 4. In the experiment, panelists remarked that cucumber fermented using freeze-dried *L. frumenti* mixed with skim milk (ratio 1:10) exhibited an acceptable overall sensory quality in comparison with the other formulations. Among the four samples, freeze-dried *L. frumenti* mixed with skim milk (ratio 1:10) had a better quality of color, odor, aroma, flavor, and saltiness, follow by the freeze-dried *L. frumenti* mixed with skimmed milk (ratio 1:2) sample. Panelists suggested that improving the crispiness of this sample could enhance its sensory appeal. Fermented cucumbers using spontaneous fermentation were described as having a pleasant taste, but their overall acceptability was negatively affected by an unappealing color, a slightly salty taste and a comparatively weaker aroma, whereas fermented cucumbers using pure culture exhibited a mild sourness. Despite its visual limitations, cucumber fermentation made from freeze-dried *L. frumenti* mixed with skim milk (ratio 1:10) was generally regarded as a favorable option for consumption.

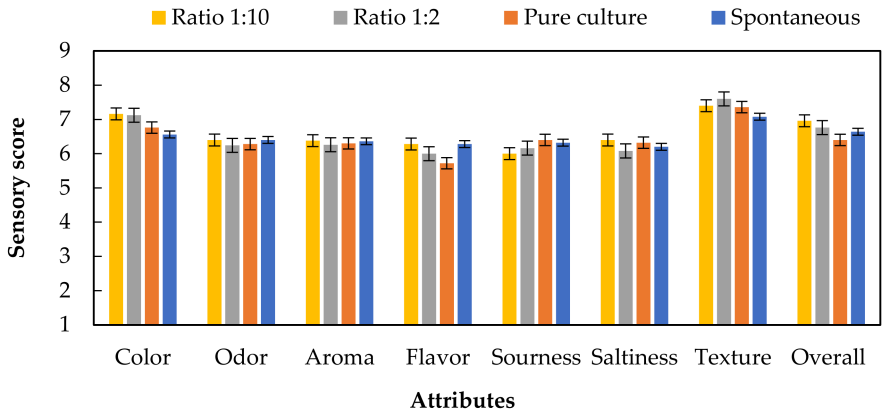



**Figure 4.** Sensory evaluation of fermented cucumbers.

## 4. Conclusions

This study showed that the type of cryoprotectant significantly influences the survival rate of lactic acid bacteria (LAB), whereas the mixing ratio showed no signification effect. Based on the results, skim milk demonstrated high effectiveness as a cryoprotectant in preserving the viability of LAB during freeze-drying, resulting in a survival rate of approximately 70%. Moreover, freeze-dried *L. frumenti* preserved with skim milk at a ratio of 1:10 improved fermentation performance in terms of physicochemical quality

and sensory acceptability. Further research is suggested to evaluate the shelf-life of the freeze-dried cultures.

**Author Contributions:** M.N.: draft and revision of manuscript, S.P.: data collection and analysis, D.B.: material preparation, S.M.: project administration and LAB identification, R.T.: experimental design, project management, review of manuscript, and supervision, Y.W.: review of manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| LAB | Lactic acid bacteria |
| *L. frumenti* | *Limosilactobacillus frumenti* |

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